REVIEW ARTICLE

Calreticulin: one protein, one gene, many functions

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The endoplasmic reticulum (ER) plays a critical role in the synthesis and chaperoning of membrane-associated and secreted proteins. The membrane is also an important site of Ca^{2+} storage and release. Calreticulin is a unique ER luminal resident protein. The protein affects many cellular functions, both in the ER lumen and outside of the ER environment. In the ER lumen, calreticulin performs two major functions: chaperoning and regulation of Ca^{2+} homoeostasis. Calreticulin is a highly versatile lectin-like chaperone, and it participates during the synthesis of a variety of molecules, including ion channels, surface receptors, integrins and transporters. The protein also affects intracellular Ca^{2+} homoeostasis by modulation of ER Ca^{2+} storage and transport. Studies on the cell biology of calreticulin revealed that the ER membrane is a very dynamic intracellular compartment affecting many aspects of cell physiology.

Key words: Ca^{2+} homoeostasis, chaperoning, endoplasmic reticulum, luminal resident protein, membranes.

INTRODUCTION

The endoplasmic reticulum (ER) is one of the largest membrane organelles in eukaryotic cells. The membrane is a site of synthesis of membrane proteins, membrane lipids and secreted proteins. It contains the largest concentration of chaperones and is an optimal environment for protein folding, modification and assembly. The ER is also considered one of the most important and metabolically relevant sources of cellular Ca^{2+} [1,2]. Ca^{2+} is released from the ER by the Ins(1,4,5)P_3 (InsP_3) receptor and/or ryanodine-receptor (RyR) Ca^{2+}-release channels and it is taken up by the sarcoplasmic/ER Ca^{2+}-ATPase (SERCA) [3–7]. Alterations in the intracellular Ca^{2+} concentration regulate a variety of diverse cellular functions, including secretion, contraction–relaxation, cell motility, cytoplasmic and mitochondrial metabolism, protein synthesis, modification and folding, gene expression, cell-cycle progression and apoptosis [1,2,8,9]. Consequently, the ability of the ER membrane to control Ca^{2+} homoeostasis has profound effects on many cell functions. The ER and its lumen contain a characteristic set of resident proteins that are involved in every aspect of the ER function [10]. In this review we focus on calreticulin, a unique ER luminal Ca^{2+}-binding chaperone implicated in playing a role in many cellular functions, including lectin-like chaperoning, Ca^{2+} storage and signalling, regulation of gene expression, cell adhesion and autoimmunity. Several excellent reviews have been published concerning the structure and function of calreticulin [11–18]. The structure and function of ER membrane and its role in control of Ca^{2+} homoeostasis has also been reviewed extensively [1,2,19–25]. The aim of the present review is to consider the latest developments in the calreticulin field, with major emphasis on the function of the protein, with focus being on the mammalian protein. Excellent review articles have recently been published on the structure and function of plant calreticulin [16], on the role of the protein in autoimmunity and viral replication [11,12,14,18] and, therefore, these subjects will not be discussed in the present review.

CALRETICULIN

Calreticulin was first isolated by Ostwald and MacLennan in 1974 [26], followed by molecular cloning of the protein in 1989 by Smith and Koch [27] and Fliegel et al. [28]. Since then, the protein has been extensively studied by many laboratories [29]. To date, cDNA and genes encoding calreticulin have been isolated from several vertebrates and invertebrates, and also higher plants ([16,29–43] and references cited therein). There is no calreticulin gene in yeast and prokaryotes whose genomes have been fully sequenced. Figure 1 shows the genomic configuration of the mouse and human calreticulin gene. The protein is encoded by a single gene [44,45], and only one species of 1.9 kb mRNA encoding calreticulin has been identified. There is no evidence for alternative splicing of the calreticulin mRNA. Another, larger, mRNA (3.76 kb in length) has been observed [28,46] but the precise identity of this mRNA is unclear.

The calreticulin gene

The calreticulin gene consists of nine exons and spans approx. 3.6 kb or 4.6 kb of human or mouse genomic DNA respectively (Figure 1) [44,45]. Human and mouse genes have been localized to chromosomes 19 and 8 respectively [44,47]. Figure 1 shows that the exon–intron organization of the human and mouse genes is almost identical. The nucleotide sequences of the mouse and the human gene show greater than 70% identity, with the

Abbreviations used: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; PDI, protein disulphide-isomerase; Grp, glucose-regulated protein; hsp, heat-shock protein; BiP, immunoglobulin-heavy-chain-binding protein; ERp, endoplasmic-reticulum protein; InsP_3, Ins(1,4,5)P_3; RyR, ryanodine receptor; SERCA, sarcoplasmic/ endoplasmic-reticulum Ca^{2+}-ATPase; ES cells, embryonic stem cells; MEF, mouse embryonic fibroblasts; $[Ca^{2+}]_{ER}$ free ER luminal Ca^{2+} concentration; NF-AT, nuclear factor of activated T-cell; TAP, transporter associated with antigen processing; β_2-m, β_2-microglobulin.

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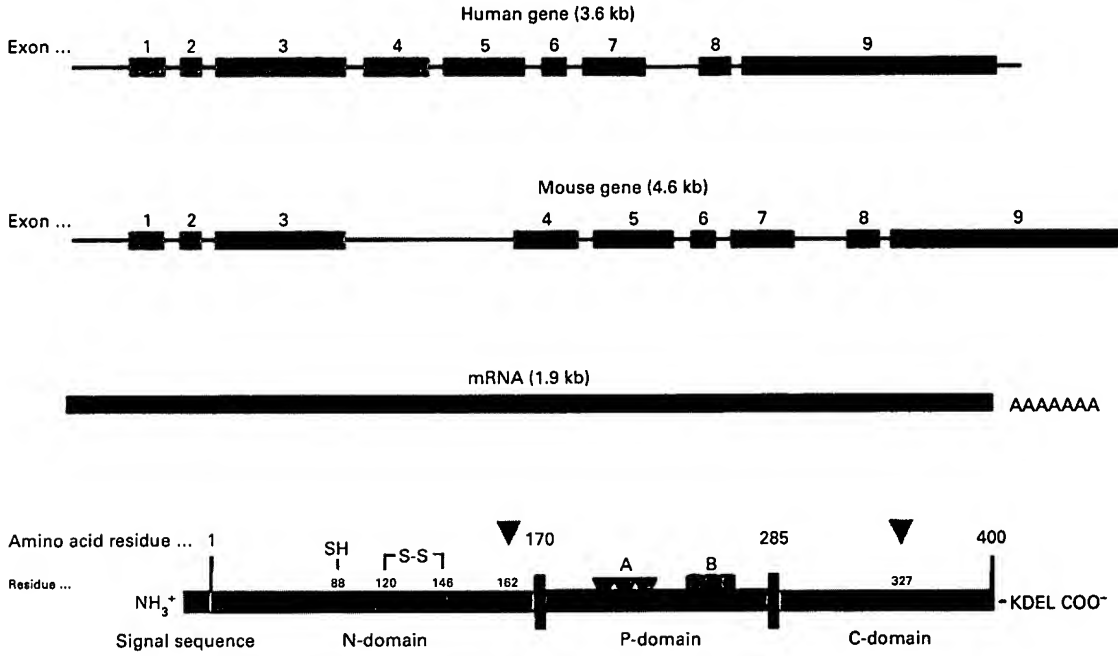

Figure 1 The calreticulin gene, the mRNA and the protein

The Figure shows a schematic representation of the genomic configuration of the human and mouse calreticulin gene, calreticulin mRNA and domain structure of the protein. Structural predictions for calreticulin suggest that the protein has at least three structural and functional domains. Exons encoding the N-domain (including the N-terminal signal sequence), the P-domain and the C-domain of calreticulin are in blue, red and green respectively. The N-, P- and C-domains are also presented in blue, red and green. The protein contains an N-terminal amino acid signal sequence (black box) and a C-terminal KDEL ER retrieval signal. The locations of three cysteine residues and the disulphide bridge in the N-domain of calreticulin are indicated. The arrowheads indicate the location of potential glycosylation sites (residues 162 and 327). Repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX) are indicated by purple triangles and purple squares respectively. The functions of calreticulin domains are listed in Table 1.

Table 1 Putative functions of calreticulin domains

(a) Structural features and function

N-domain	P-domain	C-domain
Proceeded by the N-terminal signal sequence targeting the protein to the ER lumen	Proline-rich domain	Rich in acidic amino acids
Highly conserved amino acid sequence	Amino acid sequence similarity to calnexin, calmegin and CANLUC	ER retrieval signal
Potential phosphorylation site	Putative glycosylation site (*Leishmania* protein)	Putative glycosylation site
Potential glycosylation site (bovine proteins)		Antithrombotic activity
Putative autokinase activity		Prevents restenosis
Inhibits PDI activity		Ca^{2+} 'sensor' of calreticulin–protein interactions
Suppresses tumours		
Inhibits angiogenesis		

(b) Ion binding

N-domain	P-domain	C-domain
Binds Zn^{2+}	High-affinity Ca^{2+}-binding site	High-capacity Ca^{2+}-binding site

(c) Molecules binding

N-domain	P-domain	C-domain
Binds to the DNA-binding domain of steroid receptor	Binds to a set of ER proteins	Binds a set of ER proteins
Binds to α-subunit of integrin	Strong interactions with PDI	Binds Factor IX and Factor X
Binds rubella RNA	Strong interactions with perforin	Binds to cell surface
Interacts with PDI	Lectin-like chaperone site	
Interacts with ERp57		
Weak interactions with perforin		

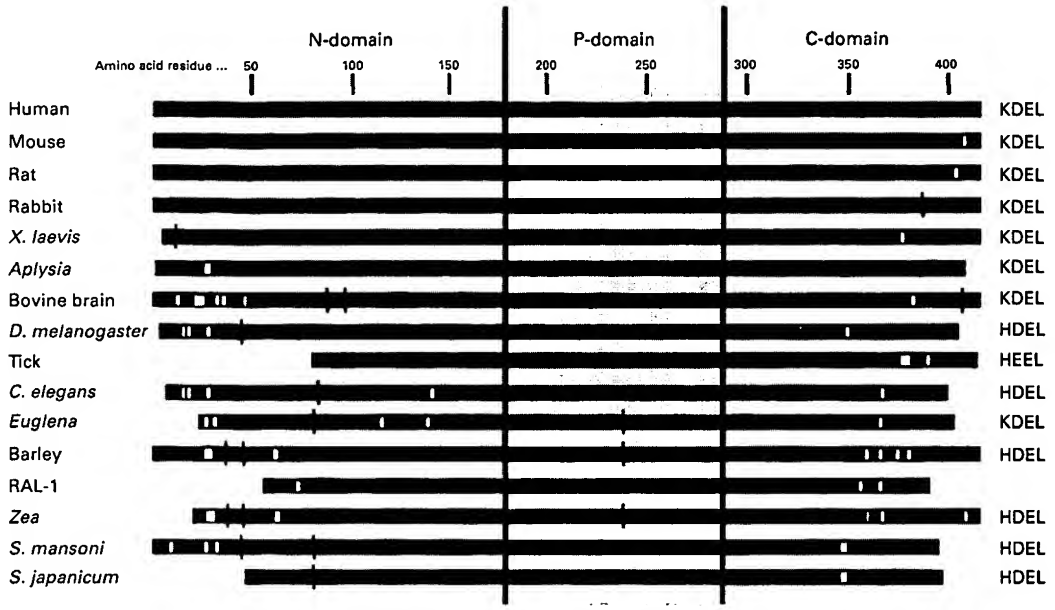

Figure 2 Comparison of the amino acid sequences of selected calreticulin proteins

Amino acid sequences of selected calreticulins were compared using MacVector version 6.0.1. software (protein matrix application). The amino acid sequences of different calreticulins are extremely similar. White regions in the black bars represent gaps in the amino acid sequence. The short vertical lines on the black bars represent amino-acid-sequence mismatches. The location of the P-domain is shaded pink. cDNAs encoding calreticulin are as follows: human (GenBank® accession numbers M32294 and M84739); mouse (X14926); rat (X79327, X53363 and S56918); rabbit (J05138); *Xenopus laevis* (South-African clawed toad) (X67597 and X67598); *Aplysia californica* (a sea slug) (S51239); bovine brain (L13462); *Drosophila melanogaster* (fruitfly) (X64461); *Amblyomma americanum* (a tick) (U07708); *Caenorhabditis elegans* (a nematode worm) (X59589); *Euglena* (a unicellular alga) (Y09816); barley (*Hordeum vulgare*) (L27349 and L27348); RAL-1 from *Oncocerca volvulus* (M20565); *Zea mays* (maize) (Z46772); *Schistosoma mansoni* and *Schistosoma japanicum* (trematode parasitic flukes) (M93097 and M80524 respectively).

exception of introns 3 and 6 [44,45], indicating a strong evolutionary conservation of the gene. In the mouse gene these introns are approximately twice the size of the corresponding introns in the human gene [44,45].

The promoter of the mouse and human calreticulin genes contain several putative regulatory sites, including AP-1 and AP-2 sites, GC-rich areas, including an Sp1 site, an H4TF-1 site, and four CCAAT sequences [44,45]. AP-2 and H4TF-1 recognition sequences are typically found in genes that are active during cellular proliferation. There is no obvious nuclear factor of activated T-cell (NF-AT) and nuclear factor κB (NF-κB) sites in the calreticulin promoter. Several poly(G) sequences, including GGGNNGGG motifs, are also found in the promoter regions of calreticulin and other ER/sarcoplasmic reticulum (SR) luminal proteins, including glucose-regulated protein 78 (Grp78) and Grp94 [8]. These motifs may therefore play a role in regulation of the expression of luminal ER proteins [8] and in ER stress-dependent activation of the calreticulin gene [45,48,49].

Depletion of Ca^{2+} stores by the Ca^{2+} ionophore A23187, or thapsigargin, an inhibitor of SERCA, induces severalfold activation of the calreticulin promoter followed by increase in calreticulin mRNA and protein levels [45,48,49]. Expression of calreticulin is also activated by bradykinin-dependent Ca^{2+} depletion of intracellular Ca^{2+} stores both *in vitro* and *in vivo* [45]. The calreticulin promoter is activated by Zn^{2+} [48] and heat shock [50]. Expression of calreticulin is also induced by viral infection [51], by amino acid deprivation [52] and in stimulated cytotoxic T-cells [53,54], further indicating that the calreticulin gene is activated by a variety of chemical and biological stresses. Since calreticulin has been implicated in a wide variety of cellular processes, the stress-dependent activation of the calreticulin gene may affect numerous biological and pathophysiological conditions.

The protein

Calreticulin is a 46 kDa protein with an N-terminal cleavable amino acid signal sequence [28,55] and a C-terminal KDEL ER retrieval signal (Figure 1). These specific amino acid sequences are responsible for targeting and retention of calreticulin in the ER lumen. Depending on species, calreticulin may have one or more potential N-linked glycosylation sites (Figure 1). The glycosylation pattern of the protein seems to be heterogeneous and does not appear to be a conserved property of the protein. The glycosylation of calreticulin is more common in plants than in animal cells [56]. Heat shock may trigger glycosylation of calreticulin [57,58]; however, the functional consequence of this stress-induced glycosylation of the protein is presently not clear. Calreticulin has three cysteine residues, and all of them are located in the N-domain of the protein. Importantly, the location of these amino acid residues is conserved in calreticulin from higher plants to that in humans [59]. Two out of three cysteine residues found in the protein form a disulphide bridge (Cys^{120}–Cys^{146}) [60], which may be important for proper folding of the N-terminal region of calreticulin.

Structural predictions of calreticulin suggest that the protein has at least three domains (Figure 1) [27,28,59]. Table 1 summarizes functional properties of these domains. The N-terminal part of the protein, encompassing the N- and P-domain of calreticulin, has the most conserved amino acid sequence (Figure 2). Careful examination of the exon–intron organization of the calreticulin gene suggests that the central P-domain of the protein

may be encoded by exons 5, 6 and 7, whereas the first four exons and the last two exons may encode the N- and C-domain of the protein respectively (Figure 1).

N-domain

The N-terminal half of the molecule is predicted to be a highly folded globular structure containing eight anti-parallel β-strands connected by protein loops. The amino acid sequence of the N-domain is extremely conserved in all calreticulins (Figure 2) [29]. The N-domain binds Zn^{2+} [61–64] and involves four of the histidine residues found in this domain [63]. The N-domain interacts with the DNA-binding domain of the glucocorticoid receptor *in vitro* [65], with rubella virus RNA [66–68], α-integrin [69] and with protein disulphide-isomerase (PDI) and ER protein 57 (ERp57) [63,70]. Interaction of this region of calreticulin with PDI inhibits PDI chaperone function [63], but enhances ERp57 activity [71]. These protein–protein interactions are regulated by Ca^{2+} binding to the C-domain of calreticulin [70]. The N-domain of calreticulin also inhibits proliferation of endothelial cells and suppresses angiogenesis [72].

P-domain

The P-domain of calreticulin comprises a proline-rich sequence with three repeats of the amino acid sequence PXXIXDPDA-XKPEDWDE (Figure 1, repeat A) followed by three repeats of the sequence GXWXPPXIXNPXYX (Figure 1, repeat B). This region of the protein binds Ca^{2+} with high affinity, and the repeats may be essential for the high-affinity Ca^{2+} binding of calreticulin [73,74]. More importantly, repeats A and B are critical for the lectin-like chaperone activity of calreticulin [75]. The P-domain of calreticulin interacts with PDI [63,70] and perforin [76,77], a component of the cytotoxic T-cell granules. The P-domain is one of the most interesting and unique regions of the protein because of its lectin-like activity and amino acid sequence similarities to other Ca^{2+}-binding chaperones, including calnexin [78], calmegin [79] and CALNUC, a Golgi Ca^{2+}-binding protein [80].

C-domain

The C-terminal region of the protein (the C-domain) is highly acidic and terminates with the KDEL ER retrieval sequence [27,28]. This domain of the protein binds over 25 mol of Ca^{2+}/mol of protein [73], binds to blood-clotting factors [81] and inhibits injury-induced restenosis [82]. Ca^{2+} binding to this domain of calreticulin plays a regulatory role in the control of calreticulin interaction with PDI, ERp57 and perhaps other chaperones [70].

CELLULAR LOCALIZATION OF CALRETICULIN

Calreticulin was originally identified as an ER/SR membrane protein [26]. Numerous studies confirmed ER localization of the protein in many diverse species, including plants [16,29,37,40,83–99]. The protein has also been localized to the cytoplasmic granules of the cytotoxic T-cell [76,77,100], sperm acrosomes [40], tick saliva [35], the cell surface [101–104], and it may even be secreted in the bloodstream [105].

Surprisingly, there is still a considerable controversy in the literature concerning the cellular localization of the protein. This is probably because of the difficulty in explaining a role for calreticulin in the control of steroid-sensitive gene expression, cell adhesion and other extra-ER functions [13,17,65,86,87, 91,99,106–117]. It was proposed that this may be due to the direct interaction between calreticulin and the DNA-binding domain of steroid receptors [65,106] and the cytoplasmic tail of α-integrin [17,69,106]. For calreticulin to bind to these molecules, the protein would have to be present in the nucleus and/or cytosol. However, to date there have been no reports on the identification of calreticulin or calreticulin-like protein in the cytosol. Calreticulin-like immunoreactivity was detected in the nucleus of some cells [94,106], in squamous carcinoma cell nuclei in response to ionizing radiation [118] or in the nucleus of the dexamethasone-treated LM(TK^-) cells [119]. However, recent studies indicate that calreticulin is not a nuclear resident protein, and earlier identification of the protein in the nucleus [94] was likely an artifact of immunostaining [86].

FUNCTIONS OF CALRETICULIN

Calreticulin has been implicated to participate in many (perhaps too many) cellular functions [12–18,65,86]. This strongly exemplifies the central role that the ER plays in a variety of cellular functions. It is not surprising, therefore, that any changes in calreticulin expression and function have profound effects on many cellular functions. However, there is a widespread agreement that calreticulin performs two major functions in the ER lumen: (i) chaperoning and (ii) regulation of Ca^{2+} homoeostasis.

CALRETICULIN, A LECTIN-LIKE CHAPERONE

Molecular chaperones prevent the aggregation of partially folded proteins, increase the yield of correctly folded proteins and assembly, and also increase the rate of correctly folded intermediates by recruiting other folding enzymes. In the last few years there has been an explosion of publications documenting that calreticulin functions as a lectin-like molecular chaperone for many proteins [13,15,16,42,71,75,95,120–153]. Calreticulin is a lectin-like chaperone similar to calnexin, an integral ER membrane protein [15,78]. Both chaperones are highly versatile being involved in the 'quality-control' process during the synthesis of a variety of molecules, including ion channels, surface receptors, integrins and transporters [15]. Although these chaperones can be considered two of the most important molecules present in a cell, we still do not comprehend fully how calreticulin assists in protein folding, how it acts jointly with other ER chaperones, and what role ER Ca^{2+} plays in these processes.

Figure 3 shows the calreticulin/calnexin cycle involved in chaperoning of glycoproteins. They bind to $Glc_1Man_9GlcNAc_2$ oligosaccharides and recognize the terminal glucose and four internal mannose moieties [75,95]. The carbohydrate attached to newly synthesized proteins in the ER lumen is of the form $Glc_3Man_9GlcNAc_2$. The glucose moieties are removed by glucosidase I and II and, if the glycoprotein is folded correctly it escapes from the folding cycle (Figure 3). However, if the glycoprotein is not correctly folded, the terminal glucose is once again attached by the UDP-glucose:glycoprotein glucosyltransferase (Figure 3), which discriminates between folded and unfolded substrates [154]. Therefore unfolded glycoproteins undergo cycles of interaction with calnexin and calreticulin. The lectin-binding site of calreticulin and calnexin is localized to the Ca^{2+} binding P-domain of the protein and the bound Ca^{2+} is essential for the lectin-like function of these proteins (Figure 3) [75]. Moreover, while glycoproteins are bound to calreticulin or calnexin, the disulphide bonds of the substrates are rearranged by the PDI activity associated with ERp57 [71,154a] suggesting that calreticulin binding to carbohydrates may be a 'signal' to recruit other chaperones to assist in protein folding. It should be emphasized, however, that monoglucosylated high-mannose carbohydrates may not be a prerequisite for substrate binding to

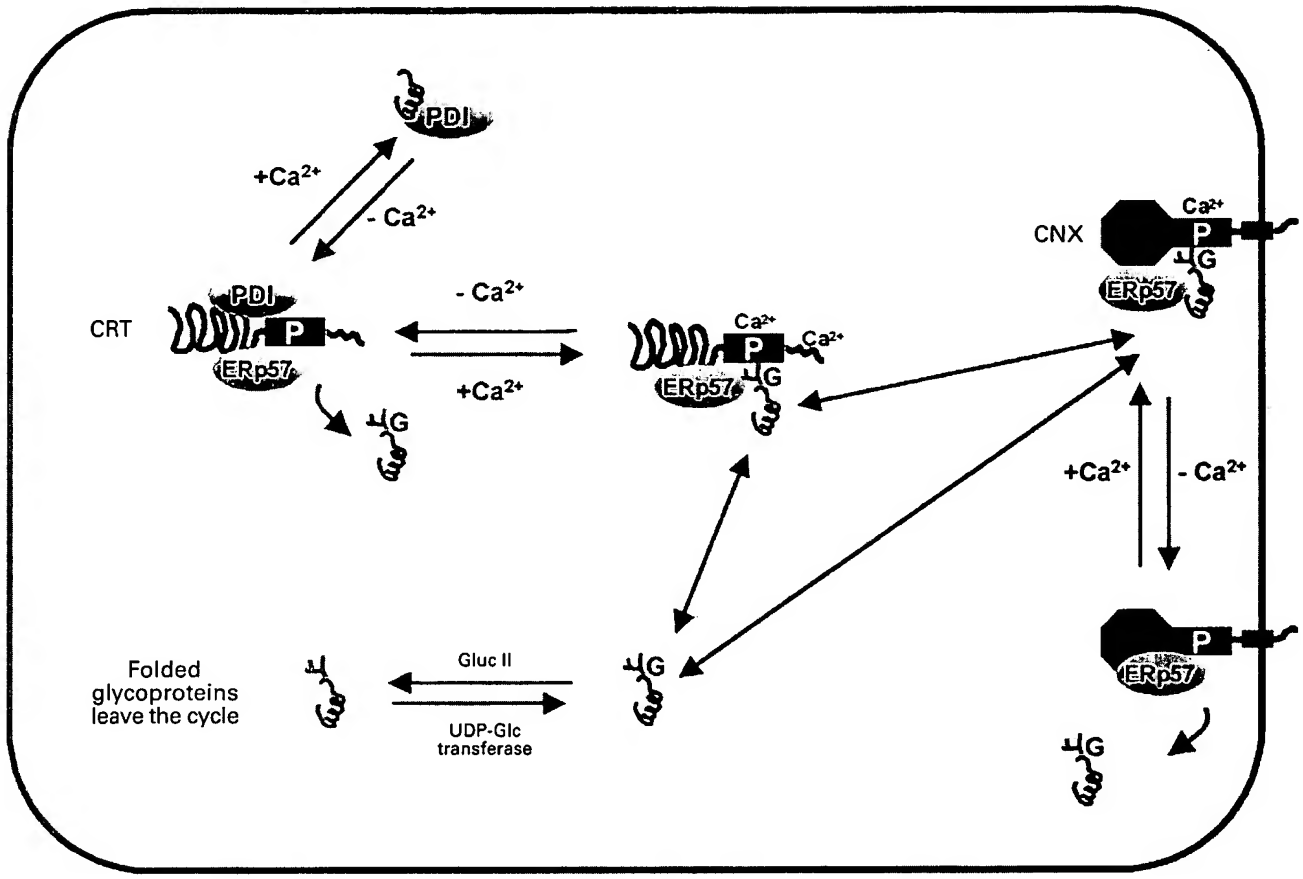

Figure 3 Calreticulin and calnexin chaperone cycle

The diagram shows interactions between ER chaperones and substrates in calreticulin/calnexin cycle in the ER lumen. Interactions between calreticulin, ER chaperones and monoglucosylated unfolded glycoproteins are regulated by Ca^{2+} as indicated. The P-domain of both calreticulin (CRT) and calnexin (CNX) bind, in a Ca^{2+}-dependent manner, $Glc_1Man_9GlcNAc_2$ oligosaccharides. Unfolded glycoproteins undergo cycles of binding to, and release from, calnexin and calreticulin. The glucose moiety is removed by glucosidase II (Gluc II) and, if the glycoprotein is folded correctly, it leaves the cycle. The terminal glucose is once again attached by the UDP-glucose:glycoprotein glucosyltransferase (UDP-Glc transferase) if the glycoprotein is not correctly folded. Calreticulin and calnexin recruit ERp57 to assist in disulphide-bond formation. Calreticulin also interacts, in a Ca^{2+}-dependent manner, with PDI, affecting PDI chaperone activity. Key to coloured portions: buff shaded ellipses, ERp57 or PDI (as indicated); black coil attached to PDI, unfolded protein; purple coil, unfolded glycoprotein; crimson rectangle with white 'P', P-domain of calreticulin; green coil, C-domain of calreticulin; light-blue portion, N-domain of calrecticulin; crimson coil, unfolded monoglucosylated glycoprotein; orange 'G', glucose residue; large purple octagon, N-domain of calnexin; small purple rectangle, P-domain of calnexin; orange coil at extreme right, the C-terminal cytoplasmic domain of calnexin.

calreticulin. For example, castanospermine and 1-deoxynojirimycin, inhibitors of the glucosidase II, do not affect association between calreticulin and Factor VIII [142] or between calreticulin and mucin [133]. Calreticulin also binds directly to PDI, ERp57, perforin, the synthetic peptide KLGFFKR and the DNA-binding domain of steroid receptors [63,69,70,76,107,108,154a], indicating that chaperone function of calreticulin may involve both protein–protein and protein–carbohydrate interactions.

Calnexin and calreticulin share many substrates and may form a link of lectin-like chaperones handing over the glycoproteins from one to the other to ensure proper folding [15,121,122,125,136,142,143,151,153,155–158]. Do calreticulin and calnexin depend on each other for proper chaperoning? The MHC class I complex is an excellent example of a molecule that employs both calreticulin and calnexin for proper folding [129,145,149,152,153,159–163]. MHC class I molecules consist of two non-covalently linked subunits, an integral membrane glycoprotein (α chain), a small soluble protein [β_2-microglobulin (β_2-m)], and a peptide of eight to eleven residues. Calnexin and calreticulin facilitate the folding of MHC class I α chains [129,145,149,152,153,159–163]. In mouse and human, α chains associate with calnexin soon after its synthesis via interactions with both immature glycans and with residues in the transmembrane domain of α chains [164]. In human α chain, β_2-m binding to α chain displaces calnexin, and the resulting α chain–β_2-m heterodimer binds calreticulin [145,163]. MHC class I expression and transport to the cell surface are not changed in calnexin-deficient cells [145,165–168], suggesting that calnexin is not essential for MHC class I synthesis and transport. These findings indicate, albeit indirectly, that calreticulin can function in the absence of calnexin. MHC class I synthesis and trafficking has not yet been investigated in calreticulin-deficient cells.

ER LUMINAL Ca^{2+} AND CALRETICULIN FUNCTION

Ca^{2+} is released from the ER by the $InsP_3$/RyR receptors [1,5–7] and taken up to the ER lumen by SERCA [4]. The ER luminal $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$) at steady state (approx. 400 μM) is two to

three orders of magnitude higher than in the cytosol [169]. Agonist-evoked Ca^{2+} release from the ER lowers $[Ca^{2+}]_{ER}$ to $< 50\ \mu M$ [169]. Ca^{2+} storage capacity of the ER lumen is enhanced by Ca^{2+}-binding chaperones. These include calreticulin, Grp94, immunoglobulin-heavy-chain-binding protein (BiP; Grp78), PDI, ERp72 and ER/calcistorin [10,24,25,170–176]. What are the consequences of Ca^{2+} release and reduction of the luminal $[Ca^{2+}]_{ER}$? Besides important effects on Ca^{2+} homoeostasis, reduction of the $[Ca^{2+}]_{ER}$ (ER Ca^{2+}-depletion conditions) leads to accumulation of misfolded proteins, activation of expression of ER chaperones [8,13,45,48,49] and ER–nucleus and ER–plasma membrane 'signalling' [8,45,48,49,177–181]. Ca^{2+} depletion inhibits ER–Golgi trafficking, blocks transport of molecules across the nuclear pore [2,182–185] and affects chaperone function [186,187]. Clearly, changes of the ER luminal $[Ca^{2+}]_{ER}$ have profound effects at multiple cellular sites, including the structure and function of the ER luminal Ca^{2+}-binding chaperones.

It is widely accepted that Ca^{2+} is a universal signalling molecule in the cell cytosol [1,2]. Can Ca^{2+} play a role of signalling molecule in the ER lumen? $[Ca^{2+}]_{ER}$ affects several processes in the ER lumen, including modulation of chaperone–substrate and protein–protein interactions [70]. For example, binding of carbohydrate to calreticulin and calnexin occurs at high $[Ca^{2+}]_{ER}$ (when Ca^{2+} stores are full) and it is inhibited at low ($< 100\ \mu M$) $[Ca^{2+}]_{ER}$ under the conditions of Ca^{2+} depletion of the stores [75] (Figure 3). Furthermore, calreticulin interacts, in a Ca^{2+}-dependent manner, with PDI [63,70] (Figure 3). Calreticulin binding to PDI results in decreased Ca^{2+} binding to calreticulin and inhibition of PDI activity [63]. Interactions between calreticulin and PDI are Ca^{2+}-dependent in a way reminiscent of the emptying and refilling of the ER Ca^{2+} stores [70] (Figure 3). Calreticulin binds reversibly to PDI at low $[Ca^{2+}]_{ER}$ ($< 50\ \mu M$), whereas the protein complexes rapidly dissociate at high $[Ca^{2+}]_{ER}$ ($> 400\ \mu M$). During emptying of ER Ca^{2+} stores, PDI and calreticulin would interact tightly, possibly causing a release of unfolded proteins from PDI, which would then be free to interact with other ER chaperones in the ER lumen. This may allow a flux of proteins to move from one chaperone to the next. A massive release of unfolded proteins from PDI and calreticulin (and perhaps other chaperones) may result in activation of the unfolded protein response without actually affecting the total concentrations of unfolded proteins in the ER lumen. Calreticulin also interacts with ERp57, a PDI-like ER luminal chaperone [70,154a]. These interactions are also regulated by Ca^{2+} [70] and affect ERp57 chaperone activity [71]. Interactions between calreticulin, PDI and ERp57 are restricted to the N-terminal region of the protein (N-domain), whereas the Ca^{2+} sensitivity is confined to the C-terminal part of the protein (C-domain) [70], suggesting that the C-domain of calreticulin may play a role of Ca^{2+} 'sensor' in the ER lumen.

Calreticulin forms structural and functional protein complexes with other chaperones. Nigam et al. [137] isolated a set of ER-resident proteins (calreticulin, BiP, Grp94, PDI, ERp72, p50 and a 46 kDa protein) that bound to a denatured protein-affinity column in an ATP- and Ca^{2+}-dependent manner. Association of calreticulin with Grp94, Grp78 and calnexin has also been reported [151]. Complexes of ER luminal chaperones are associated with maturation of thyroglobulin [130,131] and apolipoprotein B [132]. These associations and dissociation between ER luminal chaperones may be regulated by Ca^{2+} binding to calreticulin, as described for PDI and ERp57 [63,70]. Moreover, Ca^{2+} in the ER lumen may also play a role in the stability of luminal chaperones. For example, degradation of PDI and calreticulin by the protease activity of ERp72 is Ca^{2+}-dependent [188]. PDI degradation is enhanced in the presence of 1 mM Ca^{2+}, but degradation of calreticulin is inhibited by the presence of 1 mM Ca^{2+} [188]. At low $[Ca^{2+}]_{ER}$, association between PDI and calreticulin may protect PDI from degradation. When Ca^{2+} stores are full, PDI and calreticulin dissociate, resulting in PDI being more susceptible to degradation by ERp72 while calreticulin is protected. Therefore, in addition to its role in the cytosol, Ca^{2+} should be considered an important signalling molecule in the ER lumen. It is conceivable that calreticulin may be capable of sensing changes in the $[Ca^{2+}]_{ER}$ and communicating these changes to the chaperone machinery in the ER lumen.

In summary, calreticulin may be one of the central molecules in the ER lumen. The protein interacts in a Ca^{2+}-dependent manner with other chaperones and with immature glycans. The domain structure of the protein provides a unique feature enabling calreticulin to perform several functions in the ER lumen, while responding to continuous fluctuations of the free $[Ca^{2+}]_{ER}$. Calreticulin also affects Ca^{2+} homoeostasis (see below), and, in one case, the protein may even be taking advantage of its chaperone, lectin-like activity to modulate Ca^{2+} fluxes across the ER membrane [90].

CALRETICULIN AND REGULATION OF Ca^{2+} HOMOEOSTASIS

Calreticulin has two Ca^{2+}-binding sites: a high-affinity, low-capacity site ($K_d = 1\ \mu M$; $B_{max} = 1$ mol of Ca^{2+}/mol of protein) in the P-domain and a low-affinity high-capacity site ($K_d = 2$ mM; $B_{max} = 25$ mol of Ca^{2+}/mol of protein) in the C-domain [26,73]. Overexpression of the protein in a variety of cellular systems [83,85,87], including tet-on (tetracyclin)-inducible cells (K. Nakamura and M. Michalak, unpublished work), does not have a significant effect on the cytoplasmic $[Ca^{2+}]$; however, it does result in an increased amount of intracellularly stored Ca^{2+} [83,85,86]. The simplest explanation for this is increased Ca^{2+} binding to the high-capacity Ca^{2+}-binding region in the C-domain of calreticulin. It is not clear, however, if calreticulin simply binds more Ca^{2+} in the ER lumen or if it affects the level of the free $[Ca^{2+}]_{ER}$. Interestingly, Ca^{2+}-storage capacity of the ER is not changed in the calreticulin-deficient embryonic stem cells (ES) or mouse embryonic fibroblasts (MEF) [92,117]. This is likely because the other ER luminal Ca^{2+}-binding chaperones (Grp94, BiP and PDI) compensate for the loss of calreticulin. However, in calreticulin-deficient ES cells, integrin-mediated Ca^{2+} signalling is impaired, which may explain calreticulin-dependent modulation of ES adhesion [117]. Calreticulin-deficient MEF have diminished agonist-mediated $InsP_3$-dependent Ca^{2+} release from the ER [92], suggesting that calreticulin, from the ER lumen, may affect Ca^{2+} transport across the ER membrane and, consequently, the $[Ca^{2+}]_{ER}$.

Calreticulin, an ER luminal regulator of SERCA function

Ca^{2+} is taken up into the ER lumen by SERCA [1]. There are three differentially expressed genes encoding the SERCA protein [4,189]. SERCA1a and SERCA1b are expressed in fast-twitch skeletal muscle. SERCA3 is expressed in limited set of non-muscle cells [4,189]. SERCA2a is the cardiac/slow-twitch muscle isoform, whereas SERCA2b, with a C-terminal extension, is expressed in smooth muscle and non-muscle tissues that also contain high levels of calreticulin [4,29,90,189–200].

Does calreticulin affect the function of SERCA? Camacho's group carried out elegant studies on the role of calreticulin in Ca^{2+} homoeostasis utilizing the *Xenopus* oocyte model [90,201].

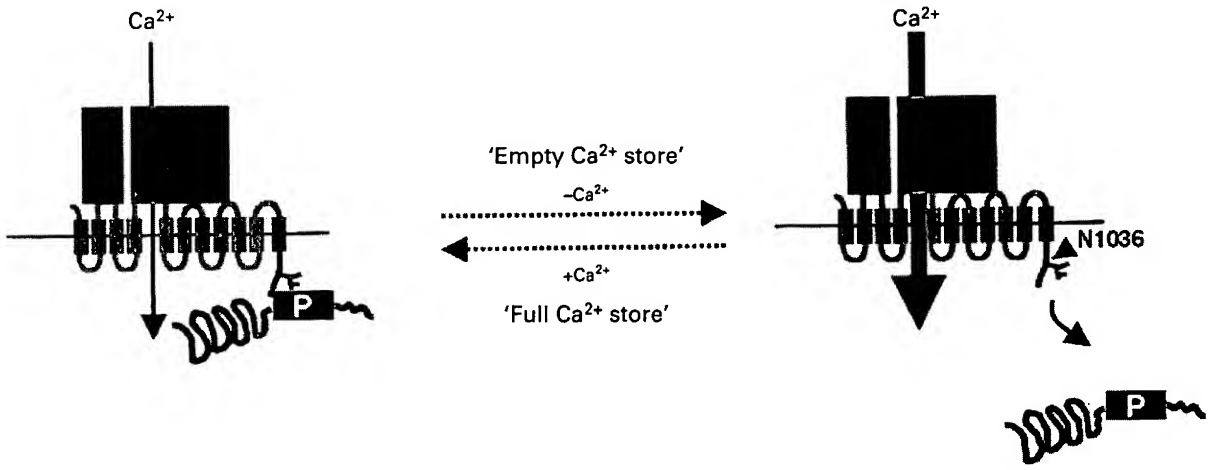

Figure 4 A hypothetical model for calreticulin-dependent modulation of SERCA function

The model shown is that proposed by Camacho's group [90]. Calreticulin may interact in a Ca^{2+}-dependent manner, with a unique C-terminal ER luminal tail of SERCA2b (orange) to modulate its Ca^{2+} transport activity and affect changes in the $[Ca^{2+}]_{ER}$. Calreticulin–SERCA2b interaction may involve a potential glycosylation site in the C-terminal region of SERCA2b [asparagine-1036 (purple N1036)]. Calreticulin binding to SERCA would reduce Ca^{2+} transport into ER lumen. In the absence of calreticulin binding to SERCA, the enzyme would exhibit full Ca^{2+}-transport activity, as represented by the thick arrow. Key to coloured portions: green rectangles, coils and cylinders, different parts of SERCA (rectangles are cytoplasmic regions of the protein, and the coils connect the cylinders, which are the transmembrane domains); orange coil, luminal tail of SERCA2b; purple coil, crimson rectangle with white 'P', the light-blue section and the green coil attached to the crimson rectangle are the same as in Figure 3.

They demonstrated that co-expression of calreticulin with SERCA2b (but not SERCA1 or SERCA2a) results in a sustained elevation in Ca^{2+} release without concomitant oscillations upon injection of $Ins P_3$ [90,201]. SERCA2b has an additional transmembrane segment (Figure 4) and a C-terminal 12-residue tail localized to the ER lumen [190–192,202,203] and containing a putative N-glycosylation site [residue asparagine-1036 (N^{1036})] [190–192]. Site-directed mutagenesis of the N^{1036} [90] or truncation studies involving N^{1036} [202] revealed that this residue is critical for calreticulin-dependent effects on SERCA2b function and for its isoform-specific functional differences. Effects of calreticulin on SERCA2b involve the P-domain of the protein, suggesting involvement of the chaperone function of calreticulin [90]. On the basis of these observations, John et al. [90] proposed that the C-terminal tail of SERCA2b may be glycosylated *in vivo* and that calreticulin modulates SERCA2b Ca^{2+}-transport activity by a direct interaction with the glycosylated C-terminal tail of the pump [90] (Figure 4). Calreticulin binding to SERCA2b may be regulated by changes in the $[Ca^{2+}]_{ER}$ reminiscent of the role of Ca^{2+} in calreticulin–carbohydrate interactions [75]. Under the conditions of empty Ca^{2+} stores, calreticulin would not interact with SERCA2b and the ATPase would exhibit full enzymic activity for efficient refilling of the stores (Figure 4). Under the conditions of full Ca^{2+} stores, calreticulin would bind to the luminal tail of SERCA, resulting in a decrease in SERCA2b activity (Figure 4). Calreticulin may play a role of a Ca^{2+} sensor for SERCA in the lumen of the ER similar to its role of a Ca^{2+} sensor for the ER luminal chaperones, as described above [70]. This is an interesting hypothesis, suggesting that a lectin-like region of calreticulin may play a dual role in the ER lumen: chaperoning of newly synthesized integral and secreted proteins and modulation of 'functional' conformations of the mature, fully functional integral (and perhaps luminal) ER glycoproteins. Although this is a very exciting hypothesis, it must be stressed that, as yet, there is no evidence for the glycosylation of SERCA2b or for its direct interaction with calreticulin.

Calreticulin and $Ins P_3$-dependent Ca^{2+} release

Once taken up by SERCA, Ca^{2+} is released from the ER by the $Ins P_3$/RyR receptors [1,5–7,204]. Calreticulin-deficient MEF cells have diminished agonist-mediated $Ins P_3$-dependent Ca^{2+} release from the ER [92], indicating that calreticulin may play a role in the functioning of the $Ins P_3$-receptor pathway. Furthermore, down-regulation of calreticulin expression with an antisense oligodeoxynucleotide results in the inhibition of bradykinin-mediated $Ins P_3$-dependent Ca^{2+} release from the ER in neuroblastoma × glioma NG-108-15 cells [205]. An attractive hypothesis is that, similar to SERCA–calreticulin interactions, calreticulin may bind to the glycosylated intraluminal loop(s) of the $Ins P_3$ receptor and modulate Ca^{2+} release [206]. The protein may also play an important role in chaperoning of both $Ins P_3$ receptor and/or molecules involved in the $Ins P_3$ pathway.

Calreticulin and Ca^{2+}-store-operated Ca^{2+} release

The increased bivalent-cation permeability in response to Ca^{2+} store depletion ('store-operated Ca^{2+} influx') may also be affected by overexpression of calreticulin [83,85,207]. However, Fasolato et al. [84] showed that overexpression of calreticulin in RBL-1 cells affected store-operated Ca^{2+} influx only when store depletion was slower than the rate of current activation [84]. When store depletion was rapid, calreticulin had no significant effects in the influx [84]. The role of calreticulin in the store-operated Ca^{2+} influx requires further investigation.

FUNCTIONS OF CALRETICULIN OUTSIDE OF THE ER

Calreticulin modulates cell adhesion [69,87,91,106,115–117], integrin-dependent Ca^{2+} signalling [117] and steroid-sensitive gene expression both *in vitro* and *in vivo* [65,86,99,106–111,113]. One major controversy in the calreticulin field concerns the

mechanisms involved in calreticulin-dependent modulation of functions outside of the ER.

Calreticulin and cell adhesiveness

The first observation pointing out that calreticulin may be involved in integrin function and cell adhesion came from an *in vitro* experiment designed to identify cellular proteins that bind to KXFF($^{K}/_{R}$)R synthetic peptide [69], a region corresponding to the conserved amino acid sequence found in the C-terminal tail of the α-subunit of integrin [208]. These early experiments revealed that calreticulin binds, in a Ca^{2+}-dependent manner, to the KXFF($^{K}/_{R}$)R synthetic peptide-affinity column [69]. It was proposed, therefore, that calreticulin may bind to the C-terminal cytoplasmic tail of α-integrin and modulate its function [17,108,115–117]. To regulate integrin activity *in vivo* by direct binding, there must be focal-contact-associated (cytoplasmic) calreticulin. However, to date, calreticulin has not been detected in the cytosol or associated with focal contacts [83,86,87].

Recent reports indicate that calreticulin may influence cell adhesion indirectly, from the ER lumen, via modulation of gene expression of adhesion-related molecules [87,91] and/or by changes in the integrin-dependent Ca^{2+}-signalling [117]. For example, the level of calreticulin expression modulates cell adhesion by co-ordinating up-regulation of expression of vinculin and N-cadherin [87,91]. Down-regulation of calreticulin causes inverse effects [87,115]. The changes in cell adhesion are also coincident with changes in the levels of protein tyrosine phosphorylation in cells differentially expressing calreticulin [91]. The abundance of phosphotyrosine in cells overexpressing calreticulin is dramatically decreased in comparison with control cells. It is well documented that protein phosphorylation/dephosphorylation of tyrosine is a major mechanism for regulation of cell adhesion [209–212]. One of the proteins undergoing tyrosine dephosphorylation in calreticulin-overexpressing cells is β-catenin (M. Opas, unpublished work), a structural component of cadherin-mediated adhesion complexes, a member of the Armadillo protein family and a part of the Wnt/Wingless signalling pathway [213]. Tyrosine-dephosphorylated β-catenin is stabilized in junctional complexes [214,215]. Phosphorylated β-catenin, when displaced to the cytoplasm, may bind specific transcription factors and translocate to the nucleus [213,216,217]. Phosphorylation of β-catenin by a serine/threonine kinase, glycogen synthase kinase-3β in a complex with axin and adenomatous-polyposis-coli protein targets it for ubiquitination and subsequent degradation [213,218–220]. Although the mechanism(s) are still elusive, it is conceivable that the effects of calreticulin overexpression on cell adhesion may be due to calreticulin effects on a signalling pathway, which includes the vinculin/catenin–cadherin protein system and may involve changes in activity of tyrosine kinases and/or phosphatases. A direct implication of this for cell–substratum interactions is that calreticulin effects may target primarily focal-contact-mediated adhesion [91].

Calreticulin and steroid-sensitive gene expression

Calreticulin binds to the DNA-binding domain of steroid receptors and transcription factors containing the amino acid sequence KXFF($^{K}/_{R}$)R and prevents their interaction with DNA *in vitro* [65,86,99,106–111,113,221]. With the exception of the peroxisome-proliferator-activated receptor ('PPAR')–retinoid X heterodimers [221], transcriptional activation by glucocorticoid, androgen, retinoic acid and vitamin D_3 receptors *in vivo* is modulated in cells overexpressing calreticulin [65,86,99,106, 109–111,113]. These are surprising findings, since calreticulin is an ER-resident protein and steroid receptors are found in the cytoplasm or in the nucleus. It is intriguing that gene expression is also regulated by another ER-resident protein, ERp61 [222]. In leukaemia cells from patients with chronic myelogenous leukaemia, ERp61 alters complex formation between nuclear proteins and regulatory regions of interferon-inducible genes [222]. What could be a physiological or pathophysiological relevance of calreticulin (ER)-dependent modulation of gene expression? Up-regulation of the calreticulin gene may correlate with increased resistance to steroids. For example, calreticulin is one of the androgen-sensitive genes in prostate cancer [223–225]. Steroid-dependent regulation of expression of calreticulin may affect differential sensitivity of patients to steroid therapies.

What could be the mechanism(s) by which calreticulin affects functions outside the ER, including cell adhesion and gene expression? Despite many years of investigation, calreticulin has not been identified in the cytosol. Cytoplasmically targeted calreticulin does not have any effect on the function of steroid receptors or cell adhesion *in vivo* [86,87,91]. Calreticulin must modulate cell adhesion and gene expression from the ER lumen [13]. The protein may participate in a signalling network in the lumen of the ER [177–181,226].

THE CALRETICULIN-DEFICIENT MOUSE

Recently gene targeting by homologous recombination was used to generate calreticulin-deficient ES cells, and their adhesion properties and Ca^{2+} handling were investigated [117]. This is the first time homologous recombination was used to knock out a gene encoding an ER luminal protein. Calreticulin-deficient ES cells had impaired integrin-mediated adhesion, as assessed by quantitative cell-attachment assays on fibronectin and laminin, fully supporting the earlier observation that changes in the expression of calreticulin indeed affect cell adhesion [69,108,117]. Calreticulin-deficient ES cells had significantly decreased integrin-mediated influx of extracellular Ca^{2+}, possibly being responsible for changes in the integrin-mediated cell adhesion [117]. Other roles of calreticulin, such as chaperone or modulation of steroid-hormone-receptor function, have not been investigated.

Since the protein is involved in a number of diverse and important functions, it was anticipated that the calreticulin knockout mouse would not be viable. Indeed, the calreticulin-deficient mouse, created by the homologous-recombination technique, is embryonically lethal at 14.5–16.5 days *post coitus* [92]. Calreticulin-deficient embryos most likely die from a lesion in cardiac development. In the adult, calreticulin is expressed mainly in non-muscle and smooth-muscle cells, and is only a minor component of the skeletal and cardiac muscle [28,89,93,96]. However, the calreticulin gene is activated during cardiac development, concomitant with an elevated expression of the protein, which decreases sharply in the newborn heart [92]. Therefore it was not surprising that calreticulin-deficient mice die from heart failure [92]. Calreticulin may belong to the family of cardiac embryonic genes and play a critical role during cardiogenesis. Grp94, another ER luminal Ca^{2+}-binding chaperone, is also up-regulated during cardiomyogenesis [227], suggesting that ER chaperones in general must play an important role in the formation of the heart.

What is the role of calreticulin in cardiac development? Cardiac development is an extremely complex process under strict transcriptional control, with the functions of many of these transcription factors depending on sustained, $InsP_3$-dependent Ca^{2+}

release [228,229]. For example, transcriptional activity of GATA-4, a transcription factor recognizing the 'GATA' motif, is enhanced severalfold by the formation of heterodimers with NF-AT, and NF-AT/GATA-4/calcineurin synergistically activate marker genes for cardiac hypertrophy [230]. Nuclear import of the NF-AT transcription factors requires dephosphorylation by calcineurin [231]. Ca^{2+} release by the $Ins P_3$-dependent pathway, but not Ca^{2+} pulses, is required to activate calcineurin and to maintain the NF-AT transcription factor in the nucleus [232]. Ca^{2+} release from the ER is impaired in calreticulin-deficient MEF, suggesting that a role for calreticulin during cardiac development likely relates to its effects on ER Ca^{2+} transport [92]. However, cardiomyocytes isolated from the calreticulin-deficient mouse beat spontaneously (N. Mesaeli and M. Michalak, unpublished work), indicating that excitation–contraction coupling and SR function are not altered in the absence of calreticulin. An important lesson that we have learned from the calreticulin-deficient mouse is that SR and ER membrane Ca^{2+} stores may be structurally and functionally distinct compartments in cardiomyocytes. Furthermore, Ca^{2+} pools that signal the developmental (or cardiac pathology) response may be distinct from those involved in excitation–contraction coupling in the SR.

An intriguing observation is that similar transcription pathway(s) (calcineurin/NF-AT/GATA) play an important role in the physiology of the immune system, cardiogenesis and cardiac pathology [233]. NF-AT is a critical component of the transcriptional regulatory apparatus of interleukin-2 in the stimulated T-cells [231]. Expression of calreticulin is up-regulated in activated T-cells [53,54,76,77,100], and the protein is targeted to the cytotoxic T-cell granules, where it forms a Ca^{2+}-dependent complex with perforin [76]. The increased level of calreticulin in activated T-cells suggests that the protein may be part of the Ca^{2+}-dependent transduction pathway(s) in stimulated T-cells, including activation of NF-AT. Therefore stress-dependent stimulation of the T-cells results in the activation of both the NF-AT and calreticulin pathways. Development of the heart as an organ must inflict a tremendous stress on cardiomyocytes. Therefore it is tempting to speculate that similar stress-induced signalling pathways are essential during cardiac development and the activation of the immune system [233].

CONCLUSIONS

Calreticulin is a unique ER-resident protein. Extensive biochemical and molecular-biological studies on the structure and function of calreticulin revealed that the protein affects many cellular functions, both in the ER lumen and outside of the ER environment. Taking this into consideration, it will be critical to understand molecular mechanisms controlling the activation of the calreticulin gene. Generation of a calreticulin-deficient mouse provides an excellent tool with which to investigate molecule mechanisms involved in multiple functions of the protein. Studies on the cell biology of calreticulin reveal that the ER membrane is not just steady machinery for protein and lipid synthesis. The membrane is a very dynamic intracellular compartment affecting many aspect of cell physiology. The ER communicates with other cellular compartments in a way reminiscent of receptor-mediated communication between the extracellular environment and the cell interior. An integral ER membrane protein may function as an intracellular receptor for the ER luminal environment (Ca^{2+} chaperones, ions, nucleotides and other molecules), which may play a role of ligand, thus affecting a variety of cellular processes as exemplified by calreticulin-dependent modulation of gene expression, cell adhesion and Ca^{2+} fluxes.

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